



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification n⁵ : C07K 7/08, 7/10, 7/02 C07K 7/18, A61K 37/24, 37/42 // C07K 7/12	A1	(11) International Publication Number: WO 92/20709 (43) International Publication Date: 26 November 1992 (26.11.92)
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(54) Title: GALANIN ANTAGONIST (57) Abstract A galanin antagonist which is a galanin receptor ligand is described. Further, new peptides Galanin(1-12)-Pro-Substance P(5-11), Galanin(1-12)-Pro-Bradykinin(2-9), Galanin(1-12)-Pro-Pro-Pro-(Leu ⁵ -Enkephalin(5-1)), Galanin(1-12)-Pro-Lys(e-NH-)Pro-(Leu ⁵ -Enkephalin(5-1)) and functional analogues and functional derivatives thereof which exhibit substantially the same galanin antagonistic effect as said peptides are disclosed. Use of the galanin antagonist, a pharmaceutical preparation comprising it and a method of treating a disorder in a mammal which depends on the physiological function of galanin at the galanin receptor are also included.		

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GALANIN ANTAGONIST

The present invention relates to a galanin antagonist which is a galanin receptor ligand. Further, it relates to the peptides Galanin(1-12)-Pro-Substance P(5-11), Galanin(1-12)-Pro-Bradykinin(2-9), Galanin(1-12)-Pro-Pro-Pro-(Leu⁵-Enkephalin(5-1)), Galanin(1-12)-Pro-Lys(ϵ -NH-Pro-(Leu⁵-Enkephalin(5-1))) and functional analogues and functional derivatives thereof which exhibit substantially the same galanin antagonistic effect as said peptides. Use of the galanin antagonist, a pharmaceutical preparation comprising it and a method of treating a disorder in a mammal which depends on the physiological function of galanin at the galanin receptor are also included.

Background

Neuro-transmitters and hormones can induce their cellular effects by binding to and activating membrane bound receptors. The neuro-peptide galanin was isolated in 1983 from porcine upper intestine and was found to contain 29 amino acid residues (Tatemoto, K., et al, FEBS Lett., 164 (1983) 124-128). The sequences of galanin from two other mammals, rat and cow, have been described (Vrontakis M. E., et al, J. Biol. Chem. 262(1987) 16755-16758; Kaplan L. M. et al, Proc. Natl. Acad. Sci. U.S.A. 85(1988) 1065-1069 and Rökaeus Å. and Carlquist M., FEBS Lett. 234 (1988) 400-406). A comparison of the peptide sequence of galanin from the mammals rat, porcine and bovine reveals that the N-terminal amino acids 1-15 are identical. Thus, it is most likely that said conserved region will be found in galanin from other mammals, including man.

Galanin has a wide pattern of distribution, often correlating with multiple neuro-formula actions exerted in a variety of different systems.

Hitherto no galanin antagonists, which are galanin receptor ligands, have been reported. A galanin antagonist would be a useful tool in determining the physiological significance of galanin and to develop pharmaceutical preparations for the regulation of the physiological function of galanin at the galanin receptor.

Description of the invention

One aspect of the invention is directed to a galanin antagonist which is a galanin receptor ligand. Thus, the antagonistic effect of the galanin antagonist according to the invention is exercised at galanin receptors. In an embodiment of this aspect of the invention the antagonist is selected from the group consisting of the peptides

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Lys-X

|

Pro-Leu-Phe-Gly-Gly-Tyr-H

wherein X represents $-NH_2$ or $-OH$ (amide or free acid), and functional analogues and functional derivatives thereof.

In the specification and claims, it is intended that "functional analogues" of the peptides of the invention i.e. should comprise shorter or longer peptides, with or

without substitution of one or several amino acid residues for other amino acid residues, as long as such analogues exhibit substantially the same pharmacological function as the peptides of the invention, i.e. are
5 galanin antagonists at the galanin receptor.

Further, it is intended that "functional derivatives" of the peptides according to the invention comprise any compound which exhibits substantially the same
10 pharmacological function as the peptides of the invention, i.e. is a galanin antagonist at the galanin receptor. Specifically, such a compound could be one which is derived from one of the peptides of the invention, but wherein one or several amino acid residues
15 are substituted for other chemical groups, i.e. organic as well as inorganic molecules or elements resulting in a "peptidomimetic".

It is believed that it is the structural conformation at
20 the galanin receptor of a peptide according to the invention which is responsible for its pharmacological function as a galanin antagonist and for its affinity to the galanin receptor (thus being a galanin receptor ligand).

25 Thus, it is believed that the functional derivatives and the functional analogues comprised by the invention should have a similar structural conformation at the galanin receptor as the peptides of the invention. The
30 surrounding of the galanin receptor could be mimicked by e.g. blood or physiological saline solution.

Another aspect of the invention is directed to the peptides

35 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

5 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Lys-X
|
10 Pro-Leu-Phe-Gly-Gly-Tyr-H

wherein X represents $-NH_2$ or $-OH$ (amide or free acid),
and functional analogues and functional derivatives
thereof which exhibit substantially the same galanin
15 antagonistic effect as said peptides.

As mentioned above, the peptides may also be named
Galanin-(1-12)-Pro-Substance P(5-11), Galanin(1-12)-Pro-
-Bradykinin(2-9), Galanin(1-12)-Pro-Pro-Pro-(Leu⁵-
20 Enkephalin(5-1)) and Galanin(1-12-Pro-Lys(ϵ -NH-)Pro-
(Leu⁵-Enkephalin(5-1))), respectively.

In the experimental part of this specification the above
listed peptides, in amide form (i.e. $X = -NH_2$), have been
25 named M15, M35, M36,A and M34,A, respectively.

Functional analogs may be peptides such as

30 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
(D-Arg)-Pro-Lys-Pro-Gln-Gln-(D-Trp)-Phe-(D-Trp)-Leu-Leu-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Gln-Phe-Phe-Gly-Leu-Met-X
35

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Ala-Leu-Ala-Leu-Ala-Leu-Ala-X

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H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Ala-Leu-Ala-Leu-Ala-X

5 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-X

10 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Ala-
-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-X

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Tyr-Gly-Gly-Phe-Leu-X

15 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X

20 wherein X represents -NH₂ or -OH (amide or free acid).

A further aspect of the invention is directed to the use
of a galanin antagonist which is a galanin receptor
ligand for the preparation of a pharmaceutical
preparation. It can be mentioned that the above listed
25 peptides are soluble in water, which facilitates the
preparation of a pharmaceutical preparation for
injection.

Yet another aspect of the invention is directed to a
30 galanin antagonist which is a galanin receptor ligand for
use in a pharmaceutical preparation.

Yet a further aspect of the invention is directed to a
pharmaceutical preparation comprising a galanin
35 antagonist which is a galanin receptor ligand as an
active ingredient, together with pharmaceutically
acceptable additive(s). Depending on the specific type of

pharmaceutical preparation to be prepared, such additive(s) should be chosen to make up the desired preparation. Suitable additive(s) for the specific type of preparation to be prepared, such as solutions for
5 injection, tablets or plasters, can be found in the U.S. Pharmacopoeia.

Thus the present invention also provides compositions containing an effective amount of compounds of the
10 present invention, including the nontoxic addition salts, amides and esters thereof, which may, alone, serve to provide the above-recited therapeutic benefits. Such compositions can also be provided together with physiologically tolerable liquid, gel or solid diluents,
15 adjuvants and excipients.

These compounds and compositions can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar
20 to other therapeutic agents. In general, the dosage required for therapeutic efficacy will range from about 0.01 to 1000 mcg/kg, more usually 0.1 to 1000 mcg/kg of the host body weight. Alternatively, dosages within these ranges can be administered by constant infusion over an
25 extended period of time until the desired therapeutic benefits have been obtained.

Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms
30 suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified or incorporated in drug delivery systems like liposomes or microspheres. The active ingredient is often mixed with diluents or excipients
35 which are physiologically tolerable and compatible with the active ingredient. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or

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the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH-buffering agents, and the like.

5

The compositions are conventionally administered parenterally, by injection, for example, either subcutaneously, intramuscularly, intraperitoneally or intravenously. Additional formulations which are suitable for other modes of administration include suppositories, 10 vagitories, intranasal aerosols, buccal formulations like adhesive tablets, gels or patches and in some cases, oral formulations. For suppositories and vagitories, traditional binders and excipients may include, for 15 example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10% preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of 20 mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained-release formulations, or powders, and contain 10%-95% of active 25 ingredient, preferably 25%-70%.

The peptide compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable nontoxic salts include the acid addition salts 30 (formed with the free amino groups) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may be derived from 35 inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino

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ethanol, histidine, procaine, and the like.

5 Still another aspect of the invention is directed to a method of treating a disorder in a mammal which depends on the physiological function of galanin at the galanin receptor, which comprises administering to said mammal a pharmacologically effective amount of a galanin antagonist which is a galanin receptor ligand. Since the physiological function of galanin at the galanin receptor is specific for each individual, the pharmacologically effective amount of a galanin antagonist which is to be administered to treat a disorder in a mammal has to be decided by a physician or veterinary on an individual bases.

15 Galanin antagonists would be useful in the regulation of at least the following:
insulin release,
growth hormone release,
20 acetylcholine release,
dopamine release,
Substance P release,
Somatostatin release,
Noradrenaline release.

25 At present, the presumed fields of medical indication are endocrinology, food intake, neurology and psychiatry: senile dementia of Alzheimer's type, schizophrenia, analgesia, intestinal diseases.

30 Preparation of a galanin antagonist according to the invention

35 Conventional methods of preparing peptides can be used to prepare the galanin antagonists of the invention, suitably modified if the antagonist is a peptidomimetic. In addition to liquid phase synthesis, conventional

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procedures for synthesizing the novel compounds of the present invention include any solid phase peptide synthesis method. In such a method the synthesis of the novel compounds can be carried out by sequentially
5 incorporating the desired amino acid residues one at a time into the growing peptide chain according to the general principles of solid phase methods.

Abbreviations used in the following:

10

	NMP	= N-Methylpyrrolidone
	HOBt	= N-hydroxybenzotriazole
	MBHA	= p-Methylbenzhydramine
	PAM	= p-Acetoxymethyl
15	Boc	= tert-Butyloxycarbonyl
	DMSO	= Dimethylsulfoxide
	DIEA	= diisopropylethylamine
	Tos	= tosyl
	OcHex	= cyclohexyl ester
20	4-MeBzl	= 4-methylbenzyl
	OBzl	= benzyl ester
	Bom	= t-Benzyloxymethyl
	Clz	= 2-Chlorobenzyloxycarbonyl
	Bzl	= O-benzyl
25	For	= formyl
	Brz	= 2-bromobenzyloxycarbonyl
	TFA	= trifluoroacetic acid
	DMS	= dimethyl sulfid
	DCM	= dichloromethan
30	DMF	= N,N-dimethylformamide

The peptides "M15", "M35" and "M36,A" of this invention were assembled in a step-wise manner on a solid support using an Applied Biosystems Model 431A Peptide
35 Synthesizer using the standard NMP/HOBt Solvent-Activation strategy on a 0.1 mmole scale (small scale).

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The peptide "M34,A" was synthesized in the same manner, except for the protective group which was ϵ -Fmoc and Boc for Lys. Accordingly one part of the peptide was synthesized using Boc-chemistry, and the other part was synthesized using Fmoc chemistry.

The functional derivatives of the invention were prepared in analogous manner.

tert-Boc-amino acids were coupled to tert-Boc-amino acid-PAM (Nova Chemical Company Ltd., UK) resin for free acid ($X = -OH$) or MBHA (Bachem Feinchemikalien AG, Switzerland) resin for amide ($X = -NH_2$) as hydroxybenzotriazole (HOBt) esters.

As individual cycle for each amino acid included deprotection of the tert-Boc-group with 50% trifluoroacetic acid in CH_2Cl_2 (DCM) for 19 min, and acylation with 10-fold excess (compared to the amount of amino groups on a resin) of the protected amino acids in a mixture of 15% DMSO in N-methyl pyrrolidone (NMP) for 35 min. Between each operation several extensive washings were performed with CH_2Cl_2 , DIEA and NMP. After each coupling acetylation (capping) was carried out using 10% acetic anhydride and 5% DIEA in NMP for 5 min.

With the exception of Boc-Lys(Fmoc) in the case of the peptide 34,A, all amino acids used were tert-Boc-protected at the N-terminal (obtained from Nova Chemical Company Ltd., UK), and their reactive side chains were protected with Tos in

Arg, OcHex in Asp, 4-MeBzl in Cys, OBzl in Glu, Bom in His, ClZ in Lys, Bzl in Ser and Thr, For in Trp, and Brz in Tyr.

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All the solvents and other reagents for automatic peptide synthesis were from Applied Biosystems.

5 The reagents used in deprotection and cleavage steps were of analytical grade and used without further purification:

1,2-ethanedithiol, acetic anhydride, trifluoromethyl sulfonic acid (TFMSA), dimethyl sulfide (DMS) and p-cresol from Fluka, diethyl ether and acetonitrile from Merck and HF from AGA Gas.

15 The fully assembled peptide-resins were dried under high vacuum overnight. Deprotection from formyl-group on Trp and benzyl-groups was performed using "low TFMSA" method. For that 100 mg of peptide-resin was treated with 2 ml of the mixture of TFMSA(10%), TFA(50%), DMS(30%), p-cresol(8%) and dimercaptoethan(2%) for 2 h at 0°C while shaking, the procedure was followed by washing with EtOH, 20 DCM, DMF, DIEA/DCM, DMF and DCM.

After drying under vacuum the peptide was cleaved from resin and deprotected by mixing of 10 ml liquid HF (containing 20% of 1:1 mixture of p-cresol and p-thiocresol) with 1 g of peptide-resin at 0°C for 60 min. 25 The resin was washed 2 times with 10 ml of Et₂O, peptide extracted with 20% acetonitrile/water and filtered. Lyophilization of the aqueous filtrate yielded the crude peptide.

30 Preparative purifications were carried out on the crude product by HPLC on reversed phase C18 column. 10 mg of crude peptide was dissolved in 1 ml of 20% acetonitrile/water and eluted with a gradient (45 min) of 35 20-60 % mixture of 0.1% TFA/acetonitrile in 0.1 &

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TFA/water at a flow rate of 2.0 ml/min. The fractions were collected according to the absorption detected at 238 nm.

- 5 Purity of the individual peptides was checked by analytical HPLC and determined to be 99 %. Molecular weights of the peptides were determined using Plasma Desorption Mass Spectrometer (PDMS) Model Bioion 20, Applied Biosystems, the calculated molecular weight
- 10 values were obtained in each case for the purified peptide.

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Table.

Purity of the individual peptides was checked by analytical HPLC and determined to be 99 %. Molecular weights of the peptides were determined using Plasma Desorption Mass Spectrometer (PDMS) Model Bioion 20, Applied Biosystems, the expected values were obtained in each case.

Galanin(1-12)-Pro-SP(5-11) amide (M15): (MW = 2199) IC₅₀ = 0.1 nM

1 20
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met amide
1 5 10 12 5 7 11

Galanin(1-12)-Pro-Spantide amide (C 7): (MW = 2827) IC₅₀ = 0.2 nM

1 13 22
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-(D-Arg)-Pro-Lys-Pro-Gln-Gln-(D-Trp)-Phe-(D-Trp)-
23 24 spantide
-Leu-LeuNH₂

Galanin(1-12)-Pro-Pro-Pro-SP(5-11) amide (M37): (MW = 2392) IC₅₀ = 40 nM

1 5 12 22
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met amide
5 11

Galanin(1-13)-Ala-Leu-Ala-Leu-Ala-Leu-Ala amide (M 38): (MW = 1970)

IC₅₀ = 0.2 nM

1 5 13 20
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Ala-Leu-Ala-Leu-Ala-Leu-Ala amide

Galanin(1-12)-Pro-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide (M 40): (MW = 1980)

IC₅₀ = 13 nM

1 5 13 20
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Ala-Leu-Ala-Leu-Alaamide

Galanin(1-13)-Bradykinin(3-9) amide (M20): (MW = 2135) IC₅₀ = 1 nM

1 13 20
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg amide
3 9

Galanin(1-13)-Bradykinin(2-9) amide (M 35): (MW = 2232) IC₅₀ = 0.2 nM

1 13 21
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg amide
2 9

Galanin(1-13)-NPY(25-36) amide (M 32): (MW = 2961) IC₅₀ = 0.05 nM

1 13 25
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr
amide
25 36

Galanin(1-12)-Ala-NPY(25-36) amide (M 88): (MW = 2935) IC₅₀ = 1 nM

1 13 25
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Ala-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr
amide
25 36

Galanin(1-12)-Pro-Pro-Pro-Leu-enkephalin(1-5) amide (M 36): (MW = 2078)

IC₅₀ = 40 nM

1 12 20
Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Pro-Pro-Tyr-Gly-Gly-Phe-Leu amide
1 5

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PHARMACOLOGICAL EXPERIMENTS

1) Galanin inhibits glucose-induced insulin release,
measured according to Ahrén, B., et al (Biochemical and
5 Biophysical Research Communications, Vol. 140. No. 3,
1986, 1059-1063). By using the same procedure as the
cited Ahrén B., et al, a peptide according to the
invention, M15, is now shown to block the galanin-
inhibited insulin release in equimolar concentration.

10

Animals and preparation of cells.

Adult obese hyperglycemic mice (ob/ob) of both sexes were
taken from a local non-inbred colony and starved
15 overnight. The animals were killed by decapitation and
the islets were isolated by collagenase isolation
technique. A cell suspension was prepared essentially as
described by Lehrnmark, Å., in Diabetologia 10, 1974,
431-438. Briefly, the islets were dissociated into single
20 cells and small clusters by shaking in a Ca^{2+} - Mg^{2+} -
deficient medium supplemented with EGTA (Ethylene glycol-
bis(beta-aminoethyl ether)N,N,N',N',-tetra-acetic acid,
SIGMA). Thereafter, the cells were incubated at 37°C, pH
7.4, overnight in 12 ml RPMI 1640 medium (Tissue culture
25 medium from SIGMA, which contains L-glutamine and does
not contain sodium bicarbonate) supplemented with 10% NU-
serum TM (Collaborative Research Inc., Lexington, Ma,
U.S.A.), 100 IU/ml penicillin, 60 µg/ml gentamycin and
100 µg/ml streptomycin. To avoid attachment of the cells
30 to the culture flasks during incubation, the suspension
was shaken gently.

Media

35 The basal medium used was a HEPES buffer (N-[2-
Hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid],
SIGMA), pH 7.4, physiologically balanced in cations with

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Cl⁻ as the sole anion. In all cases the basal medium was supplemented with 1 mg/ml albumin.

Measurements of insulin release.

5 The kinetics of insulin release were studied by perfusing pancreatic beta-cells (approx. 1×10^6) with 20 mM or 5 mM glucose mixed with Bio-Gel P-4 polyacrylamide beads (200-400 mesh, Bio-Rad Lab., Richmond, Ca, U.S.A.) in a
10 0.5 ml column. The flow rate was 0.5 ml/min and 1-3 min fractions were collected and analyzed for insulin radioimmunologically, using crystalline rat insulin as a reference.

15 Results.

After 60 min incubation in the presence of glucose (8.3 mM), galanin (10^{-7} M) completely abolished insulin release from
20 34 ± 2 μ U/ml to 3 ± 1 μ U/ml ($P < 0.001$; $N=32$). The peptide of the invention,

M 15, alone (10^{-6} M - 10^{-9} M) dose-dependently
25 counteracted the galanin-induced inhibition of insulin release.

Insulin release in the presence of glucose, galanin and M15 at 10^{-6} M, 10^{-7} or 10^{-8} was 28 ± 2 μ U/ml ($N=16$), 17 ± 4
30 μ U/ml ($N=16$), and 10 ± 3 μ U/ml ($N=16$), respectively. M15 at 10^{-9} M was without effect.

As a result of the above experiments it can be concluded that the peptide of the invention, M15, counteracts the
35 galanin-induced inhibition of insulin release in islets.

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- 2) Galanin inhibits acetylcholine release, measured according to Fisone G., et al (Proc. Natl. Acad. Sci. vol 84, 1987, 7339-7343). By using substantially the same procedure as the cited Fisone G., et al, a peptide according to the invention, M15, is now shown to counteract the inhibitory effects of galanin on the scopolamine-induced release of acetylcholine in vivo.

Microdialysis Experiments

10

- Surgery and basic methodology. Experiments were carried out with female CD-COBS (Charles River, Como, Italy) rats, weighing 200-260 g. Animals were anesthetized with equitensin (1% pentobarbital, 4% chloral hydrate). Guide cannula was implanted stereotoxically into the ventral hippocampus according to the following coordinates from the atlas of Paxinos and Watson (Paxinos G. and Watson C. (1986) The Rat Brain in Stereotoxic Coordinates, 2nd edn. Academic Press, Sydney.): 5 mm posterior to the bregma, 4.0 mm lateral to the midline, and 6.8 mm below the surface of the dura mater. A stylet was then inserted to keep the guide cannula patent until the next day. At the time the stylet was removed and replaced with a microdialysis probe (CMA 10, Carnegie Medicine AB, Stockholm, Sweden) containing a membrane having an exposed area of 3 x 0.5 mm. The microdialysis probe extended 3 mm beyond the end of the guide cannula; it was perfused at a constant rate of 2 μ l/min with Ringer's solution (NaCl, 147 mM; CaCl₂, 2.2 mM and KCl, 4.0 mM), containing 10 μ M physostigmine sulfate and adjusted to pH 7.0 with NaOH. The initial 40 min perfusate was discarded. Thereafter, perfusates were collected every 20 min during a total perfusion period of 260 min. At the end of the collection period, the samples were immediately frozen on solid CO₂ and lyophilized. Acetylcholine (Ach) content was quantified by a specific radioenzymatic method described in detail by Consolo S.,

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et al. (J. Neurochem. 48, (1987), 1459-1465.) and Wu C.F., et al (Neurobiol. Aging 9, (1988), 357-361.) and involving (a) the conversion of choline to phosphorylcholine in the presence of choline phosphokinase and ATP, (b) the enzymatic hydrolysis of acetylcholine to choline and acetic acid, (c) the reacylation of the resulting choline to [³H]acetylcholine with the addition of [³H]acetylcoenzyme-A, 99.9 GBq/mmol, and acetyl-coenzyme A:ChAT, and (d) the separation and scintillation counting of the resulting [³H]acetylcholine by extraction into tetraphenylboron-containing ketone phase by liquid-liquid ion exchange chromatography. Phosphorylation, hydrolysis and acetylation reactions were validated routinely.

The concentration of acetylcholine in each sample was calculated by linear regression based on the radioactivity of the standards (linear from 10 fmol-25 pmol acetylcholine) with the slope equal to 7800 net dpm/pmol. The coefficient of variation of the replicate perfusate samples or acetylcholine standards was approximately 3%.

In vitro recovery of acetylcholine through three dialysis tubings was determined as previously described (Wu et al., *ibid*). The average recovery was $17.6 \pm 0.5\%$.

At the termination of the release experiments, the placements of the dialysis probes were verified histologically by use of staining for Nissl substance.

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Effect of galanin and M 15 on the scopolamine-induced release of ACh, measured "in vivo" from the rat ventral hippocampus

5	Saline	2.0 pmoles
	Scopolamine	13.1 pmoles
	Scopolamine	
	+	6.9 pmoles
	Galanin	
10	Scopolamine	
	+	9.4 pmoles
	M15	

15 Galanin (1.56 nmoles) and M15 (3.12 nmoles) were injected i.c.v. 2 min before scopolamine (0.3 mg/kg, s.c.). ACh release was measured in the perfusate collected during the following 80 min, in four 20 min-fractions. The values are expressed as pmoles of ACh measured during the 20 min fraction corresponding to the peak of the

20 scopolamine-effect and corresponding to the second 20 min-fraction. The data are the means of experiments carried out in two rats (n=2).

25 As a result of the above experiments it can be concluded that the peptide of the invention, M15, counteracts the galanin-induced inhibition of scopolamine-induced release of acetylcholine in hippocampus.

30 3) Effects of intrathecal galanin and C-fiber stimulation on the flexor reflex has been described by Wiesenfeld-Hallin Z., et al (Brain Res. 486 (1989)205-213). By using substantially the same procedure as the cited reference, a peptide according to the invention, M15, is shown to have an antagonistic effect on intrathecal galanin-

35 induced facilitation of the flexor reflex. An other peptide of the invention, M35, has given similar results in preliminary experiments.

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Electrophysiological study

In acute experiments, the magnitude of the polysynaptic hamstring flexor reflex in response to activation of high threshold afferents was examined in decerebrated, spinalized, unanaesthetized rats by recording the electromyogram (EMG) from the posterior biceps femoris/semitendinosus muscles. The animals were briefly anaesthetized with Brietal®, and a tracheal cannula was inserted. The rats were mounted in a stereotaxic frame, decerebrated by aspiration of the forebrain and midbrain and then ventilated. The spinal cord was exposed by laminectomy at thoracic level and sectioned at Th₈₋₉. An i.t. catheter (PE 10) was implanted caudally to the transection with its tip on the left side of the lumbar spinal cord (L₄₋₅). The flexor reflex was elicited by test stimuli to the sural nerve or its innervation area with single electric shocks (0.5 ms, 10 mA) of sufficient strength to activate C-fibres (Wall and Woolf, 1984). In some experiments a CS (conditioning stimulus) (1 Hz, 20 s) of the same strength as the test stimuli was administered to the sural nerve.

The flexor reflex was recorded as EMG activity via stainless steel needle electrodes inserted in the ipsilateral hamstring muscles. The number of action potentials elicited during the reflex was integrated over 2 s. The integrated reflex was recorded on a Gould chart recorder (Model 2400 S). During the experiments the heart rate and rectal temperature of the rat were monitored and maintained within normal limits. The proper location of the i.t. catheter was confirmed after each experiment by laminectomy.

Galanin (Bachem, Bubendorff, Switzerland) and Somatostatin (Ferring, Malmö, Sweden) were dissolved in 0.9% saline. All peptides were injected i.t. in a volume

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of 10 ul followed by 10 ul saline to flush the catheter.

Data collection

- 5 A stable baseline reflex magnitude was established for at least 20 min before each i.t. injection or nerve CS. The effect of i.t. peptides or the nerve CS on the flexor reflex was expressed as percent change in reflex magnitude compared to baseline which was defined as 100%.
- 10 To test the interaction of galanin with other peptides or the sural CS, it was administered 1 5 min prior to the injection of the other peptides when the facilitatory effect of galanin had subsided or simultaneously with the sural CS.
- 15 The antagonistic effect of intrathecal (i.t.) M-15 on the i.t. galanin-induced facilitation of the flexor reflex was measured. The peak facilitatory effect of 30 pmol galanin was $167.0 \pm 42.8\%$ over baseline reflex magnitude.
- 20 The antagonism of M-15, injected 5-10 min after galanin was calculated as the percentage reduction of the peak facilitatory effect of galanin. Data is expressed as meant S.E.M.

25	<u>Dose of M-15</u>	<u>n</u>	<u>% antagonism</u>
	30 pmol	4	35.8 ± 20.7
	300 pmol	4	79.3 ± 8.4
	3 nmol	3	93.0 ± 3.4

30

The experiments were repeated using the peptide of the invention, M35, and similar results were achieved.

- 35 As a result of the above experiments it can be concluded that peptides of the invention counteract the intrathecal galanin-induced facilitation of the flexor reflex in a dose-dependent manner.

4) Ligand binding studies were conducted. Displacement of ^{125}I -galanin by galanin, galanin fragment (1-13), Substance P fragment (4-11), galanin fragment (1-13) + Substance P fragment (4-11) in equimolar concentrations and the peptides of the invention M15, M35 and M34, A was studied. The peptides of the invention proved to bind specifically to the galanin receptor.

Preparation of ^{125}I -monoiodo-Tyr²⁶-porcine galanin

Synthetic porcine galanin(1-29) was iodinated by chloramine-T-method to yield ^{125}I -monoiodo-Tyr²⁶-porcine galanin (specific activity 1800-2000 Ci/mmol), (as described by Land T., et al in: Methods in Neurosciences, Ed. M. Conn, 5, 1991, 225-234), and employed in ligand binding studies carried out at equilibrium.

Preparation of membrane fraction of Rin m 5F cells

The establishment and cell culture of Rin m 5F (a rat pancreatic β -tumor cell-line) cells have been described earlier (Gazdav A.F., et. al. Proc. Natl. Acad. Sci. USA, 77 (1980) 3519-3523). Briefly, the cells were grown in RPMI-1640 (Gibco) medium containing 10 % (v/v) fetal calf serum, 2.06 mM L-glutamine, 100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in 10 % CO_2 - 90 % air 37°C. They were passaged every 5 days. The cells were detached from the surface of the bottles using 0.25% trypsin containing 1 mM EDTA and centrifuged at 2000 x g for 5 min at 4°C. The pellet was exposed for 15 min to hypoosmotic 5mM HEPES buffer (pH 7.4). The suspension was centrifuged at 20000xg for 15 min, the resulting pellet was resuspended in bacitracin-containing (1 mg/mg) 5 mM HEPES-buffered Krebs-Ringer solution (137 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl_2 , 1 g/l glucose), pH 7.4, and used immediately in equilibrium binding experiments.

Displacement of ^{125}I -galanin by galanin and galanin
receptor ligands

Displacement experiments were carried out in a final
5 volume of 400 μl of bacitracin-containing (1 mg/ml)
HEPES-buffered (5 mM) Krebs-Ringer solution, 0.05 % (w/v)
of BSA (pH 7.4) in the presence of 0.1 - 0.2 nM ^{125}I -
galanin, the membrane preparation and the increasing
concentrations (10^{-12} - 10^{-6}M) of unlabeled porcine galanin
10 or of other galanin receptor ligands. Samples were
incubated for 30 min at 37°C. Incubation was terminated
by the addition of 10 ml of ice-cold HEPES-buffered
Krebs-Ringer solution, followed by rapid filtration over
Whatman GF/C filters, precoated for 5 - 6 hours in 0.3 %
15 (v/v) of polyethyleneimine solution. Filters were washed
with 10 ml of assay solution. Radioactivity retained on
the filters was determined in a Packard gamma counter.
Specific binding was defined as that displaceable by 1 μM
galanin (or appropriate concentration of a displacer).

20

The IC_{50} values of the displacing ligands were calculated
from the computer-generated IC_{50} values as described by
Land, T., et. al. (ibid).

25 Fitting of the experimental data was carried out on a
Macintosh SE by means of a nonlinear least squares method
using the Macintosh program "KaleidGraph".

30 Displacement of ^{125}I -galanin from membranes of Rin m 5F
by galanin receptor ligands

	IC_{50}
Galanin (1-29)	1 nM
M15	0.1 nM
35 Galanin fragment (1-13)	100 nM
Substance P fragment (4-11)	no displacement > 10 μM

	Galanin fragment (1-13)	}	100 nM
	+ Substance P fragment (4-11)		
	in equimolar concentrations		
	M35		0.1 nM
5	M34,A		10 nM

As is evident from the above experiments, the peptides of the invention are galanin receptor ligands.

- 10 The pharmacological experiments 1), 2) and 3) thus show that the peptides of the invention are galanin antagonists, and the experiment 4) shows that they are galanin receptor ligands. Thus, the galanin antagonists of the invention are galanin receptor ligands, unlike
- 15 other compounds which earlier have been shown to antagonize a specific effect of galanin in a specific tissue by virtue of interaction with a reaction step beyond the galanin receptor - which was involved in the biological action of galanin in that given cell type.
- 20 Such antagonism is not specific for galanin action, and is not exerted at galanin receptor. Neither it is applicable to several tissues where galanin acts, whereas the antagonists according to the present invention are bona fide antagonists in the pharmacological meaning and
- 25 exert their action at the receptor site on the outside of the cell by competing with the endogenous ligand.

Preparation of membranes from rat hypothalamus

- 30 Adult male rats (Sprague-Dawley, 180-200 g) were decapitated, the hypothalami quickly dissected and homogenized (10% mass/vol.) in 0.05 M TRIS-Cl buffer, pH 7.4. The homogenate was diluted tenfold and centrifuged at 1000 x g for 10 min. The supernatant was centrifuged
- 35 at 10 000 x g for 45 min and the pellet resuspended in 5 mM Hepes buffered Krebs-Ringer solution (137 mM NaCl, 2.68 mM KCl, 1.8 mM CaCl₂ 1 g/l glucose), pH 7.4 to yield

a final protein concentration of 1.0 - 1.5 mg/ml.

Ligand binding studies in rat hypothalamus

- 5 Displacement experiments were carried out in a final volume of 400 μ l of Hepes-buffered (5 mM) Krebs-Ringer solution, 0.05 % (w/v) of BSA (pH 7.4) supplemented with bacitracin (1 mg/ml) in the presence of 0.1 - 0.2 nM 125I-galanin, the membrane preparation from rat
- 10 hypothalami and increasing concentrations (10⁻¹² - 10⁻⁶ M) of unlabeled galanin or of other galanin receptors as ligands. Samples were incubated for 30 min at 37°C. Incubation was terminated by the addition of 10 ml of ice-cold Hepes-buffered Krebs-Ringer solution, followed
- 15 by rapid filtration over Whatman GF/C filters, precoated for 5-6 hours in 0.3% (v/v) of polyethylencimine solution. The filters were washed with 10 ml of assay solution. Radioactivity retained on the filters was determined in a Packard gamma counter. Specific binding
- 20 was defined as that displaceable by 1 μ M galanin. Rat and porcine galanin resulted in indistinguishable displacement curves with the membranes from rat hypothalami.
- 25 The IC50 values of the displacing ligands were calculated by fitting of the experimental data on a Macintosh SE by means of a nonlinear least squares method using the program "KaleidaGraph".

CLAIMS

1. A galanin antagonist which is a galanin receptor ligand.

5

2. A galanin antagonist according to claim 1, wherein said antagonist is selected from the group consisting of the peptides

10 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

15

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

20 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Lys-X

|

Pro-Leu-Phe-Gly-Gly-Tyr-H

25 wherein X represents -NH₂ or -OH (amide or free acid),
and functional analogues and functional derivatives
thereof.

3. A peptide selected from the group consisting of

30 H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-X,

35

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-
-Pro-Pro-Leu-Phe-Gly-Gly-Tyr-X,

H-Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-
-Pro-Lys-X

|
Pro-Leu-Phe-Gly-Gly-Tyr-H

5

wherein X represents -NH₂ or -OH (amide or free acid),
and functional analogues and functional derivatives
thereof which exhibit substantially the same galanin
antagonistic effect as said peptides.

10

4. A process for the preparation of a galanin antagonist
according to claim 1, characterized by

a) sequentially incorporating in solid phase the desired
amino acid residues one at a time into the growing
peptide chain, or

15

b) coupling the amino acids stepwise in a solution phase
using appropriate derivatized amino acids.

5. Use of a galanin antagonist which is a galanin
receptor ligand for the preparation of a pharmaceutical
preparation.

20

6. A galanin antagonist which is a galanin receptor
ligand for use in a pharmaceutical preparation.

25

7. A pharmaceutical preparation comprising a galanin
antagonist which is a galanin receptor ligand as an
active ingredient, together with pharmaceutically
acceptable additive(s).

30

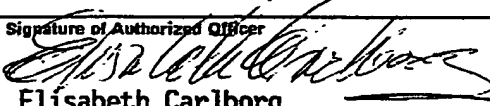
8. A method of treating a disorder in a mammal which
depends on the physiological function of galanin at the
galanin receptor, which comprises administering to said
mammal a pharmacologically effective amount of a galanin
antagonist which is a galanin receptor ligand.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 92/00316

I. CLASSIFICATION F SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: C 07 K 7/08, 7/10, 7/02, 7/18, A 61 K 37/24, 37/42/ C 07 K 7/12		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	A 61 K; C 07 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	Proc. Natl. Acad. Sci. USA, Vol. 88, pp. 10961-10965, December 1991, Tamas Bartfai et al: "M-15: High-affinity chimeric peptide that blocks the neuronal actions of galanin in the hippocampus, locus coeruleus, and spinal cord". --	1-7
P,X	Proc. Natl. Acad. Sci. USA, Vol. 89, April 1992 Zsuzsanna Wiesenfeld-Hallin et al: "Galanin-mediated control of pain: Enhanced role after nerve injury", see page 3334 - page 3337 --	1-7
P,X	Chemical Abstracts, volume 116, no. 11, 16 March 1992, (Columbus, Ohio, US), Stefan Lindskog et al: "The novel high-affinity antagonist, galantide, blocks the galanin-mediated inhibition of glucose-induced insulin secretion", see page 108, abstract 99736t, & Eur. J. Pharmacol. 1992, 210(2), 183- 188 --	1-7
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
18th August 1992	1992 -08- 21	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	 Elisabeth Carlborg	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	<p>Proc. Natl. Acad. Sci. USA, Vol. 86, December 1989 Gilberto Fisone et al: "N-terminal galanin-(1-16) fragment is an agonist at the hippocampal galanin receptor", see page 9588 - page 9591.</p> <p>---</p>	1-7
A	<p>Chemical Abstracts, volume 112, no. 1, 1 January 1990, (Columbus, Ohio, US), Kjeld Hermansen et al: "On the nature of the galanin action on the endocrine pancreas: studies with six galanin fragments in the perfused dog pancreas ", see page 77, abstract 807z, & Acta Endocrinol. 1989, 121(4), 545- 550</p> <p>---</p>	1-7
A	<p>Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), Lagny-Pourmir I. et al: "Structural requirements for galanin interaction with receptors from pancreatic beta cells and from brain tissue of the rat ", see page 72, abstract 209268y, & Peptides (Fayetteville, N.Y.) 1989, 10(4), 757- 761</p> <p>---</p> <p>-----</p>	1-7

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 8, because ^{it} ~~they~~ relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims. It is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 92/00316**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 01/07/92
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date